Puspaningtyas, International Current Pharmaceutical Journal 2012, 1(5): 92-97
http://www.icpjonline.com/documents/Vol1Issue5/01.pdf

International Current Pharmaceutical Journal

ORIGINAL RESEARCH ARTICLE OPEN ACCESS

Evaluation of the effect of red guava (*Psidium guajava*) fruit extract on tyrosinase (EC 1.14.18.1) activity by spectrophotometry

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ABSTRACT

Tyrosinase is one of the most important enzymes in melanin biosynthesis. Inhibition of tyrosinase activity will cause a decrease in melanin production. Tyrosinase inhibitory activity by ascorbic acid has been studied before. Based on reported experiments, ascorbic acid can inhibit tyrosinase activity in enzymatic reaction competitively. As an effort to find out a skin whitening agent which is effective, safe and have minimum adverse effect, the inhibitory activities of *Psidium guajava* extract was studied on tyrosinase activity. This is one of the fruits that contains high amount of ascorbic acid. To determine the efficacy of tyrosinase inhibition, L-tyrosine was used as the substrate, *P. guajava* extract as inhibitor and ascorbic acid as positive control that was measured by spectrophotometry. The optimization of method was also performed. The inhibitory kinetics was determined by measuring the absorbance of dopachrome as the end product of the tyrosinase reaction. Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) of the tyrosinase were determined by Lineweaver-Burk's plots. The K_m and V_{max} without the fruit extract were 0.315mM and 0.0265µmol/min. The K_m value with the fruit extract of 1%, 2% & 3% w/v were 0.4824, 0.698 & 0.543mM, while the V_{max} value were 0.0269, 0.0283 & 0.0255 µmol/min, respectively. Lineweaver-Burk's plots in presence of *P. guajava* fruit extract showed that the extract inhibited tyrosinase competitively. From the plots, the IC_{50} of the fruit extract was determined as 0.26mM; the control in 0.26mM concentration inhibited 56.523%. Finally, *P. guajava* fruit extract showed higher effect than ascorbic acid on Tyrosinase activity.

***Key Words*:** Inhibitory kinetics, *Psidium guajava,* fruit extract, Tyrosinase, L-tyrosine.

INTRODUCTION

Indonesia is a tropical country with abundant sunlight exposure. Sunlight had important contribution for living creature on the earth. For human, it plays important role to convert pro vitamin D_3 (7-dehydrocolesterol) on epidermis into vitamin D (Anderson, 1953; Curto *et al.*, 1999). On the contrary, sunlight may also cause negative impacts on human skin such as skin cancer and hyperpigmentation (Brown and Burns, 2002). Hyperpigmentation is caused by increased melanin production often appeared as local pigmentation or skin spot. Hyperpigmentation may be figured as blacker skin than normal skin colour (Brown and Burns, 2002). Melanin production is important to protect human skin against negative effects from ultraviolet radiation. However, high melanin concentration and melanin accumulation cause hyperpigmentation and aesthetic problems.

Now-a-days, many skin lightening products are available in the market with various active compounds to reduce hyperpigmentation and for skin lightening. Principal mechanism of skin lightening products is melanin inhibition (melanogenesis) so that skin becomes more bright and white. Melanin can be inhibited with various mechanisms such as tyrosinase activity inhibition, decreasing tyrosinase synthesis and transferm, melanosit cytotoxic inhibition etc. (Briganti *et al.*, 2003). Tyrosinase is one of the most important enzymes in the melanin biosynthesis. It is the responsible catalyst for L-tyrosine reaction in melanosit cells (Elsner and Maibach, 2000). L-tyrosine or 4-hydroxysiphenylalanin is precursor of melanin. Active compounds in skin lightening products usually inhibit tyrosinase activity such as kojic acid, hydroquinone, arbutin,

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Table 1: Reagent composition for determining kinetic parameters on tyrosinase activity.

Reagent	L-tyrosine concentrations(mM)			
	0.5	0.75	1	2
L-tyrosine	1	1	1	1
Tyrosinase	0.5	0.5	0.5	0.5
Mops buffer	1.5	1.5	1.5	1.5

Notes: All units are in millilitres.

Table 2: Reagent composition for determining kinetic and plots inhibition of *Psidium guajava* extract.

Reagent	L-tyrosine concentration(mM)			
	0.5	0.75	1	2
L-tyrosine	1	1	1	1
Tyrosinase	0.5	0.5	0.5	0.5
Mops buffer	1.4	1.4	1.4	1.4
Sample	0.1	0.1	0.1	0.1

Note: All units are in millilitres.

aloesin, ascorbic acid, cinnamic acid, and salicylic acid (Briganti *et al.*, 2003). But all of these chemical substances are not safe, for example, hydroquinone, mercury, azelaic acid, kojic acid, and Alpha Hydroxy Acids (AHAs) often causes irritation, skin burn, acute allergy, skin inflammation, skin damage and skin cancer (Lynde *et al.*, 2006; Maeda and Fukuda, 1991; Mishima *et al.*, 1988; Smith *et al.*; 1988; Nadesul, 2004). Thus, there is still room for research to find out suitable skin lightening agent preferably from natural products with minimum adverse effect (Maeda and Fukuda, 1991; Mishima *et al.*, 1988; Smith *et al.*; 1998).

Red guajava (*Psidium guajava*) is one of the fruits that contains high amount of ascorbic acid. 100g fruits of *Psidium guajava* contain an equivalent weight of 100mg ascorbic acid (Morton, 1987). Tyrosinase inhibitory activity of ascorbic acid has been studied before, and found that it can inhibit tyrosinase activity in enzymatic reaction competitively (Briganti *et al.*, 2003). The current research was designed to evaluate the effect of *Psidium guajava* extract on tyrosinase activity by determining inhibitory kinetic and kinetic plots.

MATERIALS AND METHODS

Materials

Mushroom tyrosinase (Sigma Aldirch), L-tyrosine (Merck), 0.4N HCl, Mops buffer, 1N NaOH, ascorbic acid coated CVC type A from BASF, *Psidium guajava* fruit extract, 0.01N iodine standard, amylum indicator and distilled water.

Instruments

UV-Vis Spectrophotometer (Hitachi, U-1800), micropipette (Soccorex, 100-1000µL), thermometer, pH meter, freeze dryer, analytical balance (Sartorius), volumetric flask, glass beaker.

Sample preparation

a. Preparation of tyrosinase solution

Mushroom tyrosinase 4.7mg (5370 unit) was diluted in 0.1M Mops buffer-NaOH (pH 6.5) to 100.0ml. The solutions were separated into vials and kept in freezer.

b. Preparation of L-tyrosine solution

45.5 mg L-tyrosine was diluted in 0.1M Mops buffer-NaOH to 25.0 ml (10.0 mM).

c. Preparation of *Psidium guajava* fruits extract

Psidium guajava fruit extract was collected from *Psidium guajava* fruit and homogenized by homogenizer, filtered and then dried by freeze dryer. 2.5%, 5% and 10% w/v *P. guajava* were diluted in 0.1M Mops–NaOH.

d. Preparation of ascorbic acid solution

18.49mg Ascorbic acid was diluted in 0.1M Mops buffer-NaOH to 25ml (4.20mM).

Determination of ascorbic acid concentration

0.5ml *Psidium guajava* fruit extract was diluted in 50ml distilled water and titrated by 0.01N iodine standard (16g KI/L) using amylum indicator. Distilled water was used as blank (Sudarmaji, 1996).

Maximum wavelength dopachrome optimization

This study was conducted by using Rodriquez and Flurkey method (Rodriquez and Flurkey, 1992). 0.5ml tyrosinase solution (252.39 unit/ml) and 1ml 0.1M Mops buffer-NaOH (pH 6.5) were pre-incubated at 25°C for 5 minutes. 1ml L-tyrosine solution was added and scanned for λ_{max} of dopachrome by UV-Vis spectrophotometer in 600nm-400nm. Maximum wavelength of dopachrome was determined where the highest absorbance occurred over the range.

Table 3: Result of tyrosinase activity parameters.

[L-tyrosine] (mM)	1/[L-tyrosine] (mM^{-1})	V (µmol/min)	V average (µmol/min)	1/V (µmol/min^{-1})	1/V average (µmol/min^{-1})
0.5	2	0.017 0.016	0.016	60. 453 63.660	62.057
0.75	1.33	0.019 0.019	0.019	53.691 52.689	53.190
1	1	0.021 0.021	0.021	48.048 48.241	48.145
2	0.5	0.022 0.022	0.022	44.734 44.610	44.672

Incubation time optimization

Incubation time was determined by examining dopachrome absorbance every 5 minutes up to 25 minutes (Boyer, 1993).

Determination of kinetic parameters of Tyrosinase activity

This study used Boyer and Calzyme lab modification method. 0.5ml tyrosinase solution (252.39 unit/ml) and 1.5ml 0.1M Mops-NaOH (pH 6.5) were pre-incubated at 25°C for 5 minute. Tyrosinase was added in various concentrations of L-tyrosine and then dopachrome absorbance was measured by UV-Vis spectrophotometer. 0.1M Mops buffer and NaOH pH 6.5 was used as blank. L-tyrosine concentrations were 0.5mM, 0.75mM, 1.0mM, and 2.0mM. Reagent composition for determining kinetics parameter is shown in Table 1.

Determination of kinetics and plots effects of *Psidium guajava* extract

0.5ml Tyrosinase solutions (252.39 unit/ml), 0.1ml *Psidium guajava* extract solutions and 1.4ml 0.1M Mops-NaOH (pH 6.5) buffer were pre-incubated at 25°C for 5 minute. 1%, 2%, and 3% w/v *Psidium guajava* extract concentrations in 0.1ml contain 0.029, 0.06, 0.08mM, respectively. Finally, 1ml various concentrations of L-tyrosine was added and dopachrome absorbance was measured by UV-Vis Spectrophotometer at 10 and 20 minutes. 0.1M Mops buffer-NaOH was used as blank. Reagent composition for determining kinetic *Psidium guajava* extract is shown in Table 2.

Determination of % inhibition of ascorbic acid

Procedure for determination of percentage inhibition value of ascorbic acid on tyrosinase activity was the same as *Psidium guajava* extract but ascorbic acid and only one concentration of L-tyrosine were used.

Data analysis

Determination of ascorbic acid concentration

1ml 0.01N titrant of iodine standard is equivalent to 0.88mg ascorbic acid in *Psidium guajava* extract (Sudarmaji, 1996).

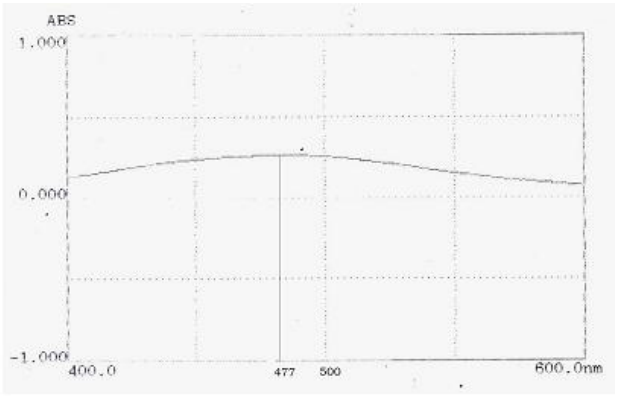

Figure 1: Result of maximum wavelength of dopachrome optimization.

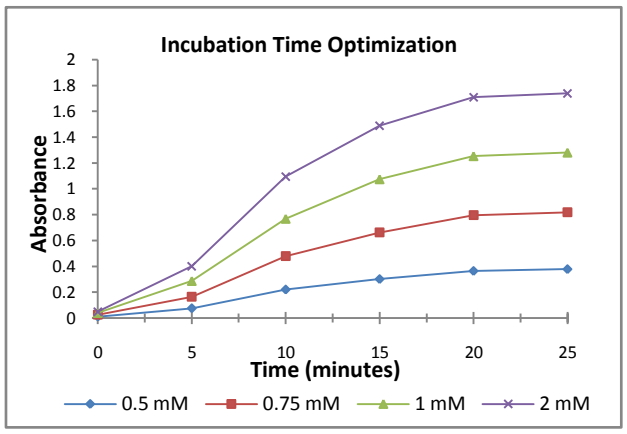

Figure 2: Result of incubation time optimization.

Table 4: Kinetic parameters of tyrosinase activity value with and without *Psidium guajava* fruit extract.

Enzymatic reaction	K_m (mM)	V_{max} (µmol/minute)
Without inhibitor	0.3150	0.0265
With inhibitor 1%	0.4824	0.0269
With inhibitor 2%	0.6980	0.0283
With inhibitor 3%	0.5430	0.0255

Determination of kinetics and plots effects of Psidium guajava extract

This study used Lineweaver-Burk curve (Boyer, 1993) that shows relationship between enzymes activity (µmol/minute) and substrate concentration ([S]). Conversion of ΔA/min value to µmol/minute was done by Lambert Beer formula:

$$c = \frac{\Delta A/min}{El}$$

c = dopachrome concentration (µmol/ minute)
$\Delta A/min$= changed absorbance per minute (minute $^{-1}$)
l = thickness of cuvette (1 cm)
E = molar concentrations of dopachrome absorbance (3600/M.cm)

Inhibition percentage of *Psidium guajava* extract and ascorbic acid was calculated by following formula (from ΔA/min value) (Boyer, 1993):

$$\%inhibition = \frac{(B - A) \times 100}{B}$$

A = rate of change absorbance value (ΔA/min) with sample
B = rate of change absorbance value (ΔA/min) without sample

RESULTS AND DISCUSSION

Determination of ascorbic acid concentrations in *Psidium guajava* extract

105.72 gram *Psidium guajava* fruit was equal to 1.65g ascorbic acid. *Psidium guajava* extract concentrations were 1%, 2%, and 3% w/v and equal to 1.56 g, 3.12 g and 4.68 g, respectively.

Determination of λ_{max} of dopachrome

Based on literature, maximum wavelength of dopachrome was 475 nm (Boyer, 1993). In this research maximum wavelength of dopachrome was found to be 477 nm (Figure 1).

Incubation time optimization

Incubation time of tyrosinase concentration 252.39 unit/ml and L-tyrosine in various concentrations 0.5, 0.75, 1.0 and 2.0mM was 20 minutes. The result was showed in Figure 2. The optimum result of incubation time was 20 minute because after 20 minutes the Michaelis-Menten curve became linear and differences of absorbance value was not significant.

Table 5: Comparison of inhibition percentage between *Psidium guajava* fruit extract as inhibitor and ascorbic acid as control.

No.	Inhibitor	Ascorbic Acid Concentration (mM)	% inhibition
1	sample 1%	0.029	17.391
2	sample 2%	0.060	26.087
3	sample 3%	0.080	30.435
4	sample IC_{50}	0.260	50.000
5	Control	0.260	56.523

Determination of Tyrosinase activity

This study employed Michaelis-Menten and Lineweaver-Burk curve to get K_m and V_{max} value. Lineweaver-Burk curve shows relationship between tyrosinase activity and L-tyrosine concentrations, resulting K_m value and V_{max} were 0.315mM and 0.0265µmol/minute, respectively. Results of Tyrosinase activity parameters are shown in Table 3. Michaelis-Menten and Lineweaver-Burk curve is shown in Figure 3 and Figure 4.

Determination of sample inhibition plot

The K_m and V_{max} value were obtained in absence and presence of *Psidium guajava* fruit extract as inhibitor in various L-tyrosine concentrations and can be seen in Table 4 and Figure 5. This curve showed cross 2 cutting on y axis. The inhibitor changed K_m value but not the V_{max} value. Inhibition type of *Psidium guajava* fruit extract on tyrosinase activity was competitive inhibition and showed in Figure 5.

Determination of % inhibition from product formed

Inhibition percentage between *Psidium guajava* fruit extract as inhibitor and ascorbic acid as control is shown in Table 5. *Psidium guajava* is one of the fruits containing high amount of ascorbic acid. Tyrosinase is the most important enzymes in the melanin biosynthesis. Inhibition of tyrosinase activity will decrease in melanin production. Tyrosinase is involved as catalysis reaction in melanin production (melanogenesis), which are L-tyrosine hydroxylation become dihydroxyphenilalanin (DOPA), and oxidation of DOPA gives dopaquinone. Kinetic and type of inhibition were determined *in vitro* where dopachrome absorbance value was taken as tyrosi-

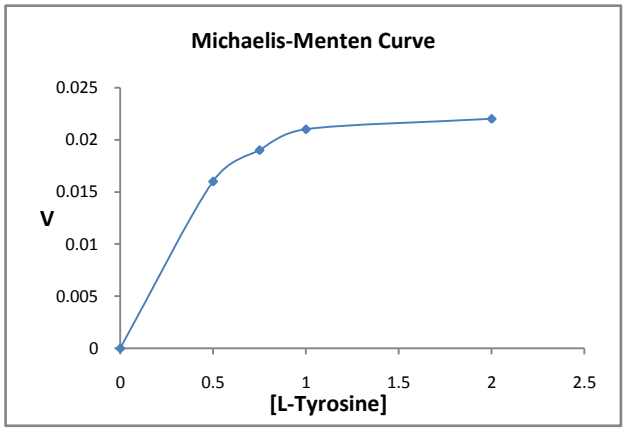

Figure 3: Relationship between tyrosinase activity and L-tyrosine concentrations by Michaelis-Menten curve.

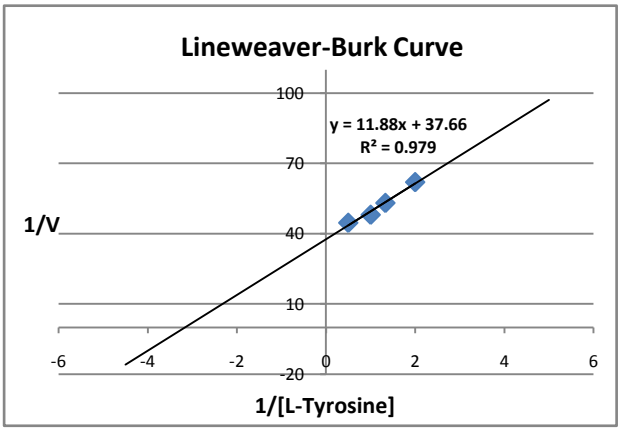

Figure 4: Relationship between tyrosinase activity and L-tyrosine concentrations by Lineweaver-Burk curve.

nase activity parameter. 1%, 2% and 3% w/v *Psidium guajava* extract contained 0.029, 0.060, and 0.080mM ascorbic acid. The IC_{50} of ascorbic acid on tyrosinase was 0.284mM (Schurink, 2007).

Determination of kinetic parameters of tyrosinase activity method used Boyer and Calzyme lab modification method. According the literature, suggested temperature is 25°C-30°C. The research condition and spectrophotometer was at room temperature (25±2°C) and at 30°C, respectively. Buffer with a pH of 6.5±0.05 was used. Optimum pH for tyrosinase catalysis reaction in the literature was found to be 6.5-7. Dopachrome has chromophore groups such as double bonds C-O in benzene rings that can absorb light. Double bonds conjugation may decrease electron transition energy and increase wavelength. 0.5 ml Enzyme was used in every procedure at a concentration of 252.39 unit/ml. According to the literature, tyrosinase concentration was 100-200 unit and absorbance value was 0.2-0.8 because it is free from noise. Determination of kinetics of tyrosinase activity was carried out at 10 and 20 minutes. Then rate of absorbance per minute (ΔA/min) was calculated. ΔA/min value showed the alteration of dopachrome concentration every minute (µmol/minute) using Lambert Beer formula. Inactivation of reaction such as added methanol, TCA (trichloroacetic acid), extreme temperature was not done because they can cause damage of dopachrome and tyrosinase enzymes. Dopachrome was counted in µmol/minute and showed tyrosinase activity in various L-tyrosine concentration.

Regression resulted by Lineweaver-Burk curve in Figure 4 is used to determine K_m and V_{max} values which were 0.315 mM and 0.027µmol/minute, respectively. The effects of *Psidium guajava* extract on tyrosinase activity was determined by Lineweaver-Burk curve and showed by cutting kinetic regression in y axis. *Psidium guajava* extract can give competitive effects on tyrosinase activity (Figure 5). The figure showed that K_m value is changed while V_{max} value remains constant. Table 5 showed that % inhibition of *Psidium guajava* extract decreases with increased L-tyrosine concentration. So, it is clear that the mechanism of enzymatic reaction was competitive (Boyer, 1993). Competitive inhibition of *Psidium guajava* extract was caused by ascorbic acid.

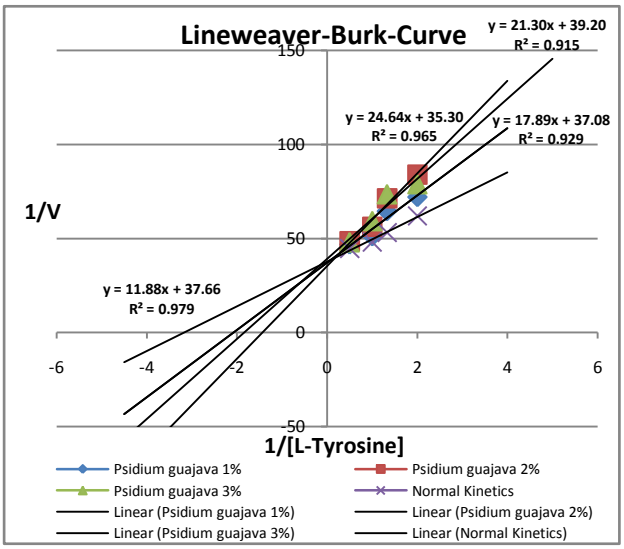

Figure 5: Relationship between enzymes kinetics with and without inhibitor.

Ascorbic acid inhibited tyrosinase activity by interaction with cuprum (Cu^{2+}) ion as cofactor in active site of tyrosinase. Ascorbic acid is an antioxidant that oxidised faster than L-DOPA in oxidation cycle and then decreases dopaquinon production (Briganti, 2003). Furthermore, to prove the inhibition mechanism of *Psidium guajava* extract on tyrosinase activity, we need L-DOPA as substrate to know inhibition mechanism of tyrosinase in cresolase or catecholase activity on the next study. Percentage inhibition of 0.260mM ascorbic acid on tyrosinase activity and 1 mM L-tyrosine concentration was 56.523%. According to the literature IC_{50} of ascorbic acid is 0.284mM (Schurink, 2007). Difference of this value was caused by usage of different substrate.

CONCLUSION

Psidium guajava extract can inhibit tyrosinase activity by competitively changing the K_m value and constant in V_{max} value. K_m values of 1%, 2% and 3% w/v concentration of *Psidium guajava* extract were 0.4824, 0.6980 and 0.5430, respectively, whereas V_{max} values were 0.0269, 0.0283 and 0.0255µmol/min, respectively.

ACKNOWLEDGEMENT

The author expresses gratefulness to Jember University, for providing fund for the research. The author is also grateful to the Department of Chemistry, Jember University for providing the facilities to execute this study.

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